Proposed Adaptations to the OECD Draft Guidance Document

Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure

PHASE 1 Test Procedure

30 July 2015

Introduction

This document integrates modifications developed at the University of Florida, Gainesville, FL, USA (Schmehl et al. 2015) to the OECD Draft Guidance Document for Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure (OECD 2014). The proposed adaptations are based on a method developed in France (Aupinel et al. 2005, Aupinel et al. 2007) and on methods described in the COLOSS Beebook (Crailsheim et al. 2013) and provides a tool for testing the toxicity of chemicals on honey bee survival when fed to bees during the larval stage of development. The proposed adaptations have not been previously tested in outside laboratories. The aim of this document is to provide a protocol to be followed by twelve US and European laboratories to evaluate control survival in honey bees reared *in vitro* on an artificial diet.

Phase 1 Experimental Design Overview

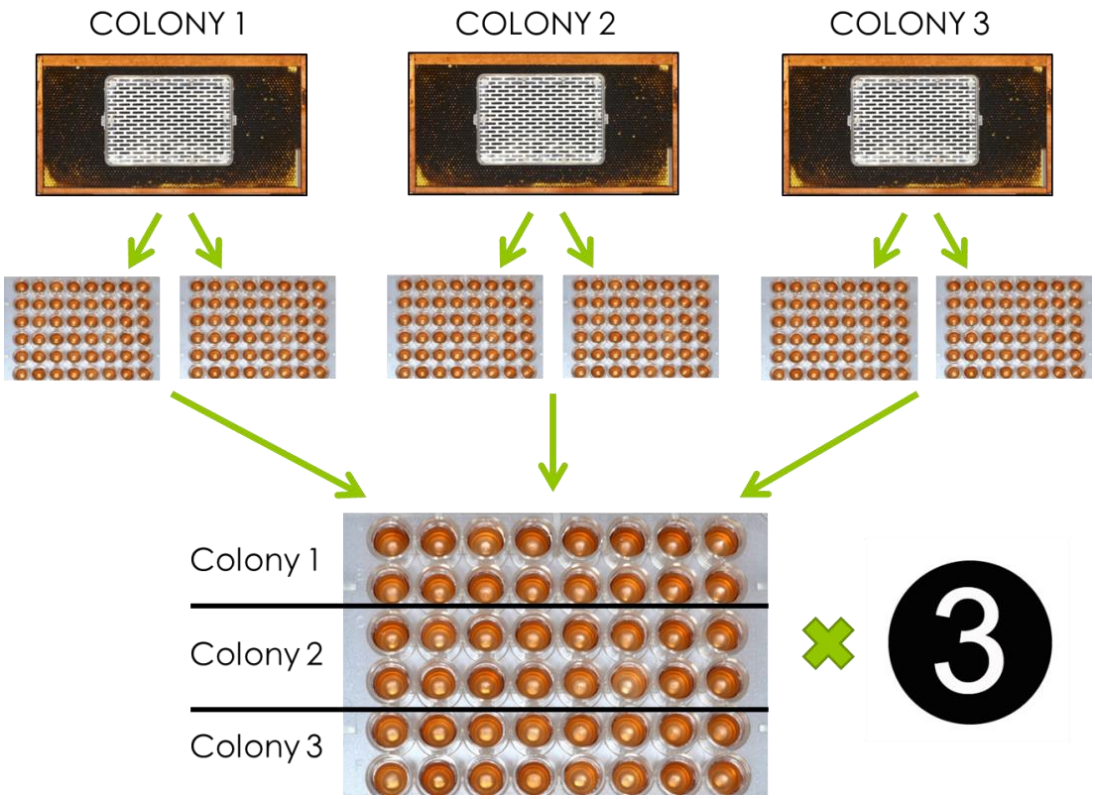

Figure 1. Overview of the Phase 1 experimental design.

Queens will be confined to empty brood comb on one frame per colony in at least 5 colonies for a 24 hour period. After 24 hours, the queen will be released from the queen excluder cage. Once released, the cage will be placed back without the queen to prevent the queen from relaying additional eggs in the same location. After a period of 75 hours (3 days and 3 hours after the queen is released), three frames (one frame/colony for three colonies) with young larvae (1^{st}-2^{nd} instar) are transferred to the laboratory. Two plates of 48 larvae/plate will be transferred from the frame of each of the three colonies to the larval plates (grafted in excess, totalling 96 larvae/colony for three colonies). On day 2 (D2, two days after grafting), the larvae will be allocated by transferring 16 cell cups with healthy larvae from each colony to a new sterile culture plate so that each plate contains 48 larvae from the three colonies (16 larvae X 3 colonies = 48 larvae). The study will be conducted in triplicate, resulting in a total of three plates with 16 larvae/colony on each plate (Fig 1).

Principle of the Test

On day 0 (D0) of the study, young larvae (between 1^{st}-2^{nd} instar) of a synchronized age are taken from the comb of three colonies and individually placed into 48-well plates where they are fed a standardized amount of artificial diet through day 5 (D5, five days post grafting). Prepupal honey bees (only the bee) are transferred to a new culture plate after their diet is fully consumed (D6-D8, six to eight days post grafting respectively). The bees' development progresses from young larvae (between 1^{st} and 2^{nd} instar) to prepupae (Fig 2) in the larval plate and continue their pupation in the pupal plate until adult emergence (Fig 3). Mortalities of the bees are recorded on day 2 (D2, two days after grafting) through to day 8 (D8, seven days after grafting), D12 and adult emergence (D18-21). The test ends when all bees have emerged or died.

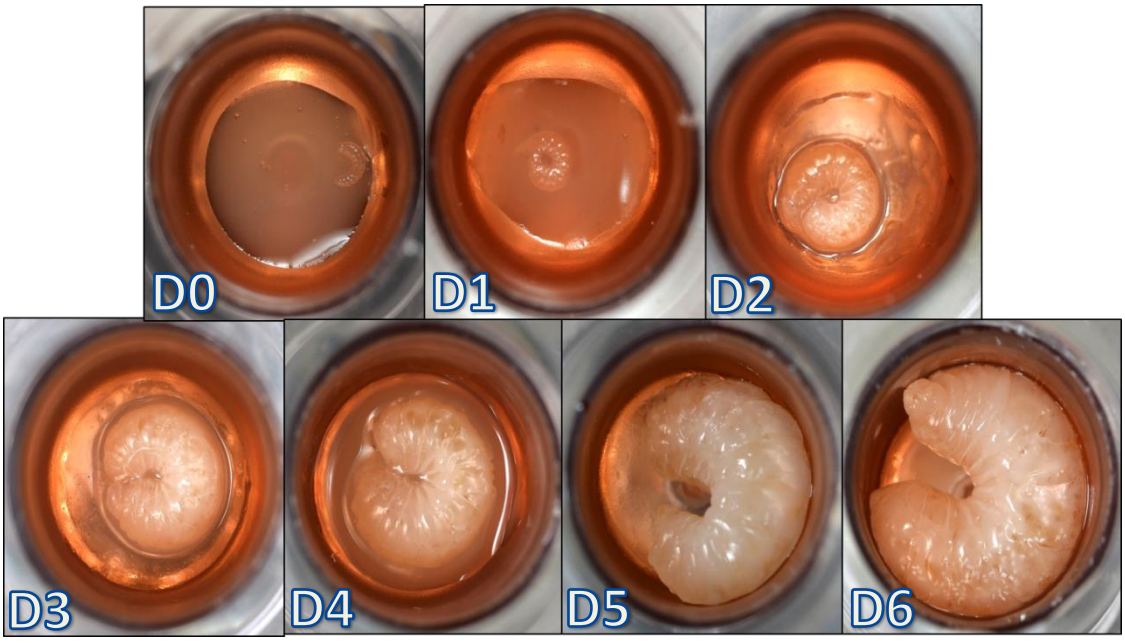

Figure 2. Progression of larvae on day 0 at time of grafting (top left image) through day 6 at the time of the pupal transfer (bottom right image).

Figure 3. Progression of the prepupae on day 6 (top left image, immediately after transfer to pupal plate) through adult emergence (bottom right image).

Supplies

A list of tools and supplies necessary for the proposed adaptations can be found in 'Appendix A' and 'Appendix B'. To provide uniformity in the testing conditions for Phase 1, several items are required to be standardized (including manufacturer) among testing laboratories and are listed in 'Appendix A' and includes 48-well sterile culture plates (with lid), brown plastic queen cell cups, Kimwipes, D-fructose, D-glucose, yeast extract, royal jelly (US only- Stakich, Lot#14020701, distributed at UF rearing workshop), and 0.22 µm filtered H_2O. Any deviations from the standardized list must be recorded on the Phase 1 questionnaire. A required non-standardized item list is provided in 'Appendix B'. All consumables must be new for Phase 1 testing. Culture plates, brown queen cell cups, Kimwipes, grafting tools, pipettes, and tools for mixing the diet must be sterilized by UV light, autoclave, or sterilizing solution (eg. 70% ethanol) prior to beginning the proposed adaptations.

Phase 1 Test Acceptance Criteria

All data and completed questionnaires generated from Phase 1 testing must be submitted regardless of poor control survival or adverse outcomes including, but not limited to, environmental conditions, equipment failure, or experimental error. The information generated from Phase 1 will be used to propose validity criteria for judging the acceptance and quality of the larval toxicity test, repeated exposure and compare the proposed methodology adaptations to the OECD guidance document.

Schedule for *in vitro* rearing methodology

A detailed feeding and maintenance schedule is provided in Appendix C and should be referenced daily while conducting the larval rearing assay.

Procedure

Preparation (All supplies are listed in Appendix A and B)

1. Larval plates are prepared by placing brown plastic queen cell cups within each well of a 48-well polystyrene cell culture plate (Fig 4). At this time the plate can be sterilized by placing the entire plate including the lid under UV light for at least 15 minutes. Alternatively the plate and queen cell cups can be sterilized in a sterilizing solution prior to placing the cells within the plates. Be sure to always leave the lid on the plate once the larval plate is sterilized. Three additional sterile culture plates will need to be set aside for D2 (two days after grafting) when the larva from each of the three colonies will be allocated within a single plate and performed in triplicate (see Fig 1). NOTE: Do not place dental rolls or sterilizing solution within the wells as this will negatively impact the survival of larvae when the lid is placed on top of the plate.

Figure 4. Prepared larval plate for *in vitro* honey bee rearing assay.

2. Pupal plates are prepared by placing a 2 cm x 1 cm piece of Kimwipe within each well of a 48-well polystyrene cell culture plate (Fig 5). The Kimwipe needs to be flat against the bottom of the cell with the edges of the Kimwipe extending upwards against the side of the well. Sterilize the pupal plate by placing under UV light for at least 15 minutes. Be sure to always leave the lid on the plate once the pupal plate is sterilized. Set pupal plate aside until D6 when prepupal- staged bees begin to consume all of their diet and are ready to be transferred from the larval plate to the pupal plate.

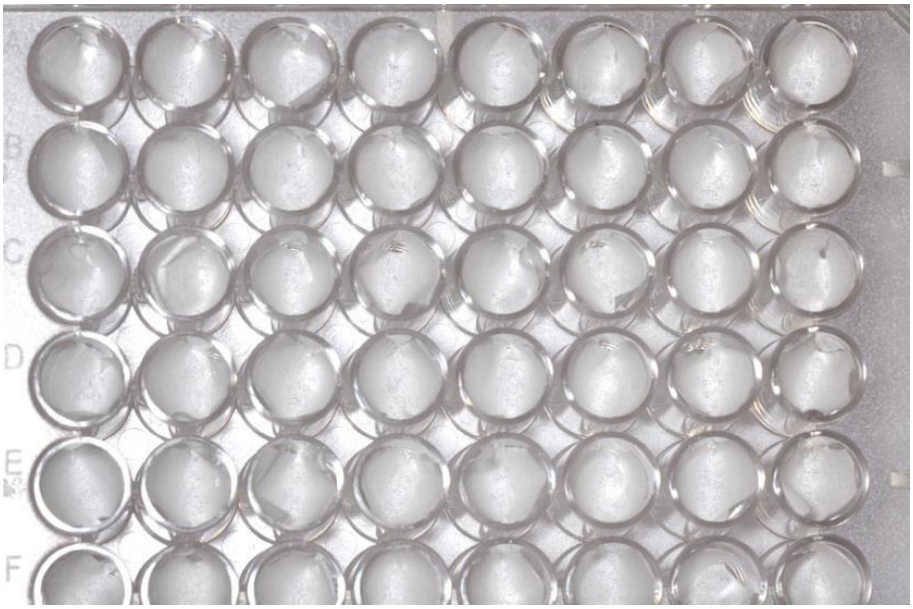

Figure 5. Prepared Pupal Plate for *in vitro* honey bee rearing assay.

3. Prepare tools for larval grafting and pupal transfer. If using a plastic Chinese grafting tool for grafting larvae from the frame to the larval cup, trim the long edge of one side of the grafting tool filament to allow the filament to fit more easily into the brood cell (Fig 6A). Prepare at least three grafting tools to allow sterilization of tools during grafting. Be sure that the white plastic plunger comes in complete contact with the filament when the plunger is depressed. To prepare the pupal transfer tool from the plastic Chinese grafting tool, remove the white plastic plunger and spring from the tool (Fig. 6B).

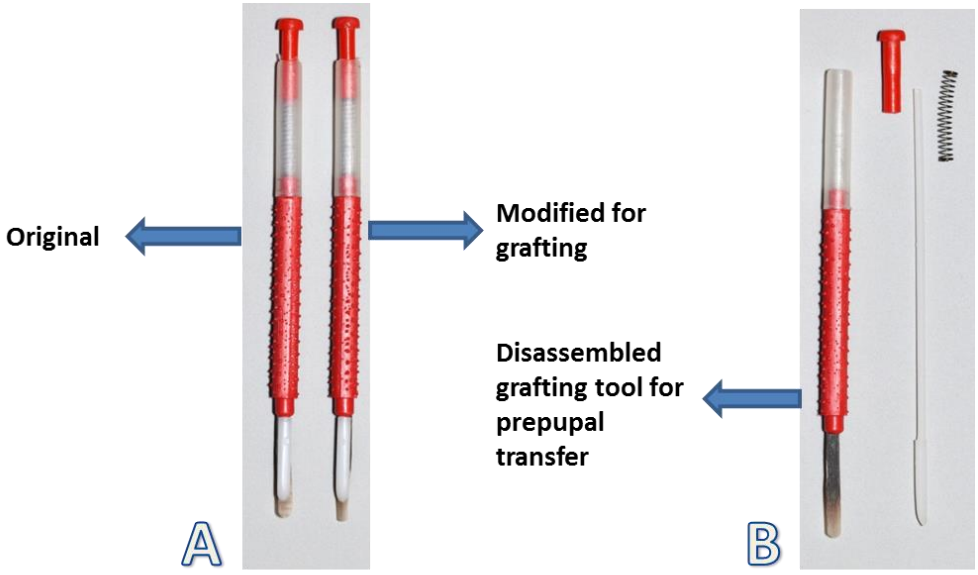

Figure 6. Modified plastic Chinese grafting tool (A) and pupal transfer tool (B).

4. Sterilize all tools, materials, and supplies by using a UV light, 70% ethanol, or an autoclave. Tools include grafting tools, beakers and tools for mixing the diet. Be sure any tools that are used can be adequately sterilized (i.e. no wooden Chinese grafting tool permissible).

5. Prepare at least two desiccators and one incubator by clearing all interior surfaces with a 10% bleach solution before each new round of larval grafting. Do not begin the rearing protocol until all surfaces are adequately dried.

6. Place desiccators within an incubator set at 35°C and should not vary more than 0.5°C (Fig 7). A data logger should be placed within each desiccator to record the temperature and humidity of the rearing environment.

Figure 7. Incubator with larval and pupal desiccator chambers each equipped with a data logger to monitor and record temperature and relative humidity.

7. Prepare K_2SO_4 (~160 g K_2SO_4 to 1 L H_2O) and NaCl (~400 g NaCl to 1 L H_2O) supersaturated salt solutions by mixing the salts in 45°C tap water.

8. One desiccator will be maintained at 94% R.H. and used for the larval stage of development, while the second desiccator will be maintained at 75% R.H. and used for the pupal stage of development. Place the K_2SO_4 salt solution within the tray of the larval desiccator and the NaCl salt solution within the tray of the pupal desiccator.

9. Prepare the larval diet within one week of Phase 1 testing (stored at 4°C until use). The larval diet is fed a diet of water, sugars, yeast and royal jelly in the amounts/proportions listed on Table 1. The provided adaptations to the composition of the diet increases the water content and reduces the likelihood of the diet drying out while grafting and feeding the larvae. The amounts provided in the table are enough to feed a minimum of 400 larvae throughout the larval development stage.

Table 1. Amount and percentage of diet components in the larval diet necessary to feed approximately 400 larvae.

Diet component	**Amount of diet components (g)**			**Percentage of diet components in total diet**		
	Diet A	Diet B	Diet C	Diet A	Diet B	Diet C
Royal Jelly	4.43	4.30	25.00	44.25%	42.95%	50.00%
Glucose	0.53	0.64	4.50	5.30%	6.40%	9.00%
Fructose	0.53	0.64	4.50	5.30%	6.40%	9.00%
Yeast Extract	0.09	0.13	1.00	0.90%	1.30%	2.00%
Water	4.43	4.30	15.00	44.25%	42.95%	30.00%
Total	10 g	10 g	50 g	100%	100%	100%

10. Allow all components to warm to room temperature prior to mixing the diet. Prepare the diet within a hood to prevent contamination. Do not shake to mix as this incorporates air into the diet and reduces the diet density. Mix in the following order by hand stirring or vortex.

a. Filter room temperature H_20 using a 0.22 µm membrane syringe.

b. Measure the amount of filtered H_2O.

c. Add the two sugars (Fisher, D-fructose and Fisher, D-glucose) to the water and mix by hand stirring or vortex.

d. Add the yeast (Bacto) and mix all ingredients until completely dissolved

e. Add the royal jelly and mix completely until dissolved. Please note that the US Phase 1 testing laboratories are all using the same source of Stakich royal jelly (Lot # 14020701R).

11. Once the diet is mixed, weigh 1 mL of diet to confirm that the density of the diet is approximately 1.10-1.15 g. Record the weight of 1 mL of diet for diets A, B, and C on the Phase 1 questionnaire.

12. On the day of grafting, place 20 µL of diet A within each of the 48 cell cups, place the lid on the plate, and place the plate within the larval desiccator to prewarm the plate and diet to 35°C. This step must be performed within a hood. It is mandatory to use gloves when handling the plate and touching any interior parts of the desiccator.

13. Sanitize the hood or bench top that will be used as a grafting station. Place a light source and heat source at the grafting station. If using a clean hood, a space heater turned on the low setting (~31°C) and positioned ~15 cm from the edge of the frame is preferable for warming the grafting environment. If using a bench top, do not use a space heater, as this will circulate particulates in the air while grafting. Alternatively, place the larval plate directly on top of a heated pad or block that can be set between 34-35°C. The diet will not dry out during grafting and feedings.

14. Place one small beaker (~100 mL) filled with 70% ethanol and one small beaker (~100 mL) filled with H_20 at the grafting station. The ethanol and water will be used to sterilize the grafting tools periodically (~ every 8 larvae) during grafting.

Collection of larvae

15. A honey bee queen from a queen-right colony is confined to a section of the wax comb using a queen exclusion chamber (Fig 8). Cage at least five queens across five different colonies to increase the probability of having three suitable frames for grafting (Fig 1).

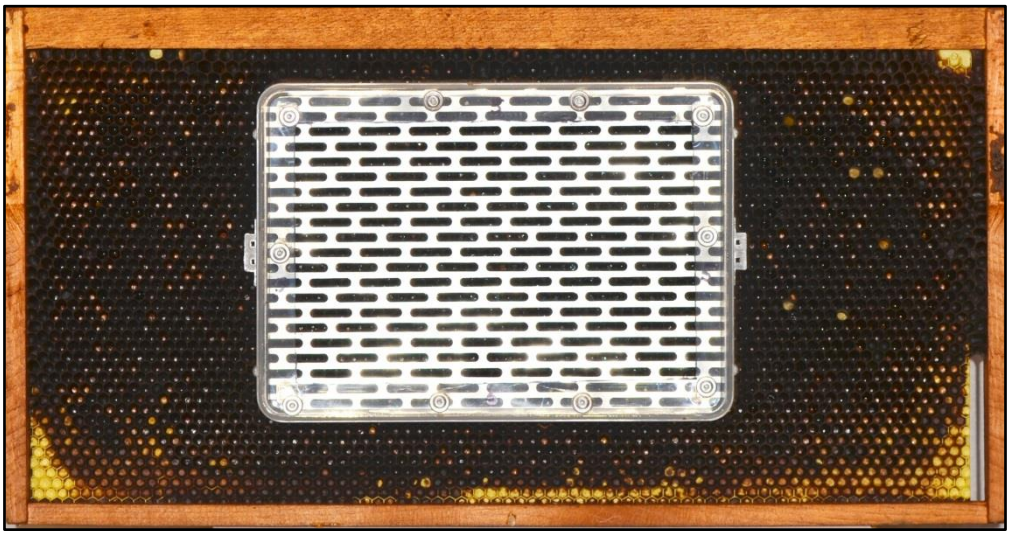

Figure 8. Queen exclusion chamber on frame of young larvae prior to transporting to the laboratory.

16. Remove the queen from the cage after a period of 24 hours and confirm that the queen has laid eggs in the cells. Replace the queen excluder cage in the original position.

17. On day 0 (day of grafting), frames of young larvae (1^{st}-2^{nd} instar) are transported to the laboratory 75 hours after the queen was removed from the exclusion chamber. Be sure to transport larvae within a heated box (eg. Nuc or full-sized hive body with two telescoping lids equipped with

a heat pack heated according to manufacturer's directions. A heated transport box should be used regardless of the distance traveled between the apiary and testing laboratory.

18. Upon return to the laboratory, immediately place the frames for grafting into an incubator set at 35°C until the time of grafting. Do not keep frames within incubator for more than 1 hour prior to the start of grafting.

Larval grafting and maintenance

19. Remove one larval plate (using gloves) and place in proximity to a heat source (reference #13) within the grafting station. Remove a frame from one of the colonies and position the frame at your grafting station at a slight angle (5-10°) to facilitate the transfer of larvae from the frame to the larval plate.

20. Insert the tip of the grafting tool into the brood cell, gently lift the larva upwards out of the cell, and place on the surface of diet A within the cell cup (Fig 9). Preferentially graft larvae in wells which contain a milky white pool of diet underneath the larvae. The larvae are easier to remove from the wells by inserting the grafting tool at the back of the C-shaped larvae (opposite of the open side of the larvae, see Fig 9B). Discard any grafting errors. Do not graft larvae that have noticeable defects. The day of grafting is referred to as day 0 (D0).

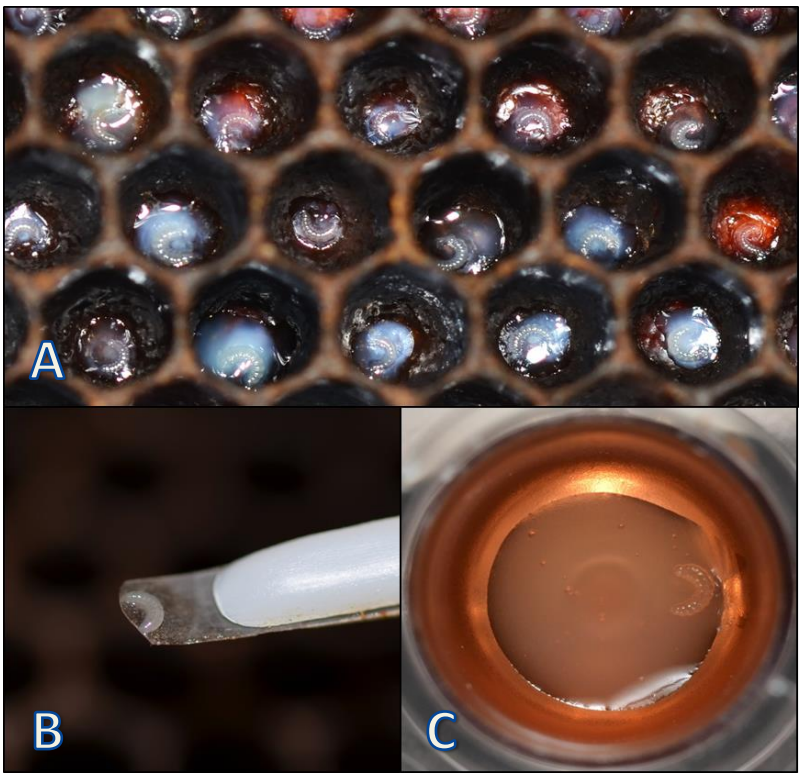

Figure 9. Young larvae in comb (A), grafted larva on tip of plastic grafting tool (B) and larva placed within cell cup (C).

21. Repeat until each cell cup across the 48-well plate is occupied with a grafted larva. Once the plate is completely occupied, confirm the presence of a larva in each well and immediately place the

plate (covered with lid) within the incubator. Each plate should take no more than 20 minutes to complete.

22. For Phase 1 testing, repeat steps #19-21 until a total of 2 plates (96 larvae) are grafted from each colony for a total of 3 colonies (Fig 1). Do not disturb the larvae until D2 (48 h post-grafting).

23. On D2, place three empty sterile culture plates in the incubator to pre-warm to 35°C.

24. Remove the two plates from "Colony 1" and record larval mortality.

25. Select 48 of the healthiest (diet consumed, no sign of disease, standardize size) larvae across the two plates and transfer 16 larvae in the cell cups into each new culture plate for a total of three plates.

26. Repeat steps #24-25 for "Colony 2" and "Colony 3" until 16 larvae from "Colony 1", 16 larvae from "Colony 2", and 16 larvae from "Colony 3" are placed on each of the 3 plates (Fig 10).

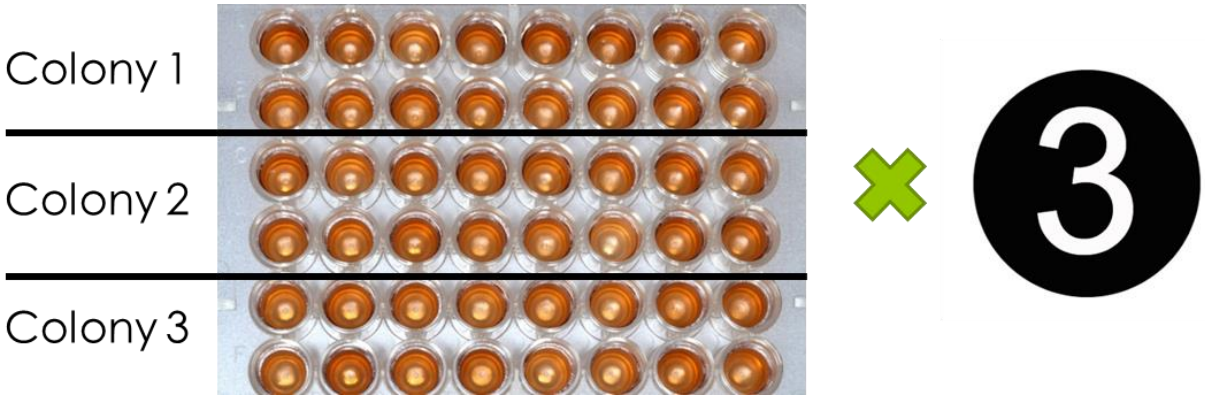

Figure 10. On day 2 (two days post-graft), 16 larvae per colony for each of the three colonies are transferred to a single new culture plate. Repeat until there are a total of three plates representing larvae from the three colony replicates.

27. Once the larvae are transferred On D2, place 20 µL of diet B (Table 2) into each well by gently dispensing the diet along the walls of the cell cup. Be extremely careful not to drown the larva by placing the diet directly on the top of the larva.

28. Monitor and record mortality on D3, D4, and D5 and remove any dead larvae by lifting the entire cell cup out of the well and disposing in the trash.

29. Feed the larvae 30 µL, 40 µL, and 50 µL of diet C on D3, D4, and D5 respectively (Table 2). Never remove any diet from the cell cups.

Table 2. Schedule of larval feeding during the *in vitro* protocol.

Time after Grafting	**Diet**	**Amount of diet (µL)**
DO, t= 0	A	20
D1, t = 24 h	n/a	0
D2, t = 48 h	B	20
D3, t = 72 h	C	30
D4, t = 96 h	C	40
D5, t = 120 h	C	50

Pupal transfer and monitoring

30. On D6 (six days post-graft), the bees begin to finish consuming the provisioned diet and they transition into the prepupal stage of development. Once the bees consumed the entire diet, the prepupae are transferred into the pupal plate (Fig. 5). Do not transfer the larvae prior to the diet being fully consumed. Be sure not to delay the transfer of the larvae to the pupal plate once the diet is fully consumed. Bees generally consume all of the control diet within D6 and D7, but some bees may be delayed and only transferred on D8 (see steps #31-35 below). Be sure to monitor and record mortality on D6-D8

31. Each pupal plate should be labeled to correspond with a larval plate so each bee is transferred into the same position. For example, a prepupae on Larval Plate 1 in position A5 will be transferred to Pupal Plate 1 in position A5. There will be a total of three pupal plates to correspond with the three larval plates.

32. To transfer the prepupae to the pupal plate, remove the cell cup containing the prepupae and gently loosen the prepupae from the walls of the cell using the modified pupal transfer tool (Fig 11). Once the prepupae is loosened, tilt the cell cup downward at a ~45° above the target well and allow the prepupae to gently fall into the well of the pupal plate. The position of the prepupae in the well is not important and the prepupae will orient itself correctly.

33. Once all the prepupae that have consumed all of their diet are transferred (i.e. not all prepupae may be transferred on D6), place the lid onto the pupal plate into the pupal desiccator (75% R.H.). Place the corresponding larval plate back into the larval desiccator (94% R.H.). Repeat for all the larval plates until the transfer for D6 is completed.

34. On D7, repeat steps #31-32 until all prepupae that fully consumed their diet are transferred.

35. On D8, repeat steps #31-32 until all prepupae that fully consumed their diet are transferred. Any bees that have not fully consumed their diet after D8 are considered dead and discarded.

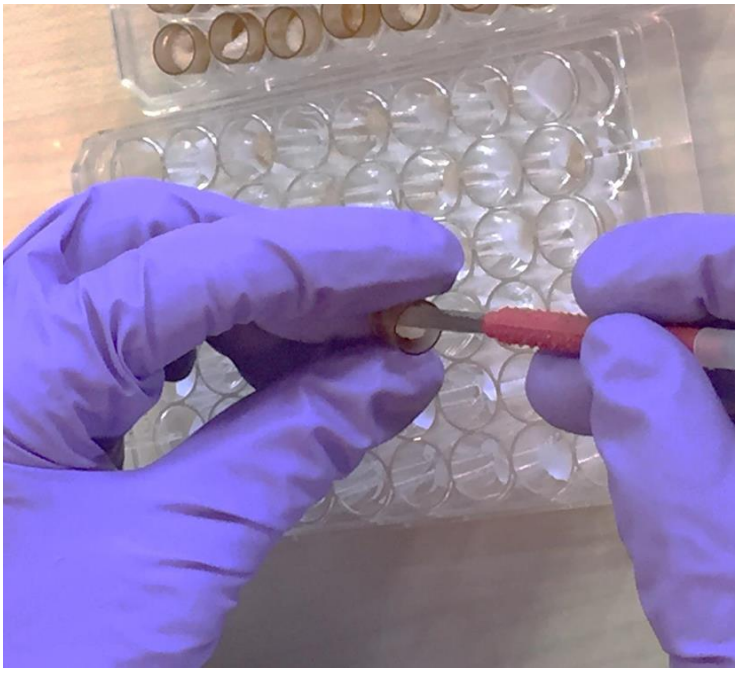

Figure 11. Transfer of prepupae to the pupal plate.

36. Pupal mortality is monitored on D12. Any dead pupae are removed from the wells and discarded. Do not uncover the plate and place in an emergence chamber. The pupal plate remains covered throughout adult eclosion.

37. Monitor and record adult emergence from D18-D21. Plates should be monitored for emerging adults within the wells at least once daily. An eclosed adult is defined as: 1) differentiated wings, and hairs with leg movement (early stage of eclosion), 2) Active walking movement within pupal cell (mid-stage of eclosion), and 3) Fully eclosed adult bee that died of starvation (late stage of eclosion).

38. Remove dead pupae and emerged adults from the wells and replace the plate cover. Any pupae that have not reached adult eclosion by D21 are considered dead.

Reportable Data (Phase 1 standardized data sheet will be provided to each participant)

1. The number of mortalities due to grafting on D2

2. The number of mortalities on D3-D5 (larval), D6-D8 (prepupal), D12 and D18 (pupal)

3. The number of bees pupating on D6-D8

4. The number of adults emerged on D18-22

Acknowldegments

Development of the proposed adaptations, pictures, tables 1 and 2, and appendix C were provided by the Jamie Ellis laboratory located at University of Florida, Gainesville, FL, USA.

References

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Crailsheim, K., R. Brodschneider, P. Aupinel, D. Behrens, E. Genersch, J. Jutta Vollmann, and U. Riessberger-Galle. 2013. Standard methods for artificial rearing of. Apis mellifera:1-16.

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Appendix A

Required (standardized) Equipment/Supplies ***Use specified item unless unavailable***	**Minimum Quantity**	**Notes**
48-well culture plates, sterile	1	Falcon, #353230
Brown plastic queen cell cups	1	Mann Lake LTD, #QC-110
Kimwipes	1	Kimberly Clark, #06-666A
D-fructose	1	Fisher, #L95-500
D-glucose	1	Fisher, #D16-500
Yeast extract	1	Bacto, #212750
0.22µm filtered water	4 L	Can use PVDF membrane filter or water from Millipore (or equivalent)
Stakich Royal Jelly	50 mL vial	Distributed at UF workshop, not standardized for European testing

Appendix B

Required non-standardized Equipment/Supplies	Minimum Quantity	Notes
Desiccator	2	Recommend Nalgene Acrylic Desiccator Cabinets (Item # 5317-0070, 5317-0120, 5317-0180)
Incubator	1	Must have capacity for at least 2 desiccators
Refrigerator	1	Storage of royal jelly, larval diet
Microwave	1	Warming of heat pad used during transport
Heat source for during grafting	1	A space heater is preferable if using a hood, but a hot plate or heating coil is satisfactory as long as the temperature can be controlled. Set between 30°-35°C
Data Logger	2	Onset HOBO UX100-011 recommended
Honey bee colonies	5	A minimum of five queens should be caged to ensure that a minimum of three queens have adequate larvae at the time of grafting
Queen excluders	5	Full frame or push-in cage
Heat packs	1	Used during frame transport to laboratory, clay preferable
Frame transport box	1	Capacity for transporting at least three frames
Metal laboratory tools or vortex	Variable	Used for mixing diet
Analytical balance	1	Weighing diet components, confiming larval diet density
Grafting tools	Variable	Plastic Chinese Grafting Tool (grafting, prepupal transfer) or a metal tool (eg. Dental tool) for the actual larval grafting
Pipette	1	Use repeater or variable volume pipette

Sodium Chloride	1 container	Maintain R.H. of pupal desiccator at 75%
Potassium Sulfate (K2SO4)	1 container	Maintain R.H. of larval desiccator at 94%
Nitrile Gloves	1 box	
Disinfectant spray	1	For cleaning benchtops/hood/incubator/desiccators (eg. 10% bleach)
Autoclave/UV light	1	Sterilization of plates/tools
Hood	1	Preparation of diet/plates
Light source	1	Used to illuminate frame during grafting

Appendix C

In vitro rearing time reference points. For the "age of bee from t = 0," 0 is the midpoint of the time the queen was caged. Once the queen is released, the eggs she laid are 0 ± 12 h old if she was caged for 24 hours. We discuss the tasks performed at each time point in the "Task performed" column. We also provide a sample time schedule that aligns with the mentioned tasks and puts all tasks associated with the rearing protocol at reasonable times of the day.

Time d (h) recognizing grafting as t=0	**Time d (h) since initiating *in vitro* rearing protocol**	**Age of bee d (h) from t = 0. All times are ± 0.5 d or 12 h.**	**Task performed**	**Sample daily time schedule**
-4 (-99)	0 (0)	-0.5 (-12)	Cage queen	10:00
-3 (-75)	1 (24)	0.5 (12)	Release queen	10:00
-2 (-51)	2 (48)	1.5 (36)	N/A	
-1 (-27)	3 (72)	2.5 (60)	N/A	
0	4 (99)	3.625 (87)	Graft /Feeding (Diet A)	13:00
1 (24)	5 (123)	4.625 (111)	Inspection	13:00
2 (48)	6 (147)	5.625 (135)	Feeding (Diet B) /Inspection	13:00
3 (72)	7 (171)	6.625 (159)	Feeding (Diet C) /Inspection	13:00
4 (96)	8 (195)	7.625 (183)	Feeding (Diet C) /Inspection	13:00
5 (120)	9 (219)	8.625 (207)	Feeding (Diet C) /Inspection	13:00
6 (144)	10 (243)	9.625 (231)	Pupal Transfer/Inspection	13:00

7 (168)	11 (267)	10.625 (255)	Pupal Transfer/Inspection	13:00
8 (192)	12 (291)	11.625 (279)	Pupal Transfer/Inspection	13:00
9 (216)	13 (315)	12.625 (303)	N/A	13:00
10 (240)	14 (339)	13.625 (327)	N/A	13:00
11 (264)	15 (363)	14.625 (351)	N/A	13:00
12 (288)	16 (387)	15.625 (375)	Inspection	13:00
13 (312)	17 (411)	16.625 (399)	N/A	13:00
14 (336)	18 (435)	17.625 (423)	N/A	13:00
15 (360)	19 (459)	18.625 (447)	N/A	13:00
16 (384)	20 (483)	19.625 (471)	N/A	13:00
17 (408)	21 (507)	20.625 (495)	N/A	13:00
18 (432)	22 (531)	21.625 (519)	Inspection/Adult Emergence	13:00
19 (456)	23 (555)	22.625 (543)	Inspection/Adult Emergence	13:00
20 (480)	24 (579)	23.625 (567)	Inspection/Adult Emergence	13:00
21 (504)	25 (603)	24.625 (591)	Inspection/Adult Emergence	13:00